Comparative Evaluation of Three Diphosphonates: In Vitro Adsorption (C-14 Labeled) and In Vivo Osteogenic Uptake (Tc-99m Complexed)

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We have investigated the in vitro adsorption of three C-14-labeled diphosphonates on calcium phosphate. The three are 1-hydroxy[1-^{14}C]ethylidene diphosphonate (C-14 HEDP), [^{14}C]methylenediphosphonate (C-14 MDP), and hydroxy[^{14}C]-methylenediphosphonate (C-14 HMDP). All three adsorbed significantly more, per mole of calcium, on amorphous calcium phosphate than on crystalline hydroxyapatite. Among the three diphosphonates, C-14 HMDP adsorbed—on both amorphous and crystalline calcium phosphate—to a greater degree than did the other two bone-seeking agents. Moreover, when HMDP was complexed with Sn(II) and Tc-99m, it produced a significantly higher uptake of Tc-99m, per mg of calcium, in an isolated in vivo site of osteogenesis. The mechanisms of adsorption are discussed relative to the hydroxyl group on the diphosphonate, to the solubility of the calcium salts of the diphosphonates, and to the form of the calcium phosphate. These studies form a working rationale for the clinically observed high contrast obtained with Tc-99m HMDP between normal bone and soft tissue, and between normal and abnormal bone.

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The effective detection of subtle abnormalities of bone (such as an early metastatic lesion) by bone-seeking agents (*1,2*) depends upon the stability of the Tc-99m ligand complex (*2*), upon its passive diffusibility (*3*), and upon the specific affinity of the ligand portion for the site of abnormal osseous metabolism (*3,4*). Such an abnormal site is usually characterized by the deposition of calcium phosphate (CaP) of low density and high hydration (*5*) in an amorphous state (*6*), such as that found in embryonic (*7*) and newly forming bone (*5,8*).

The present study is an examination in vitro of the adsorption reaction (*9*) of various C-14-labeled diphosphonates with both synthetic amorphous (CaP_{am}) and crystalline CaP_{cr} to simulate adsorption at abnormal hypermetabolic bone and normal bone, respectively. In addition, the uptake of Tc-99m complexed with these same diphosphonates in the presence of Sn(II) is examined in isolated sites of osteogenesis (*10,11*) and CaP deposition.

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MATERIALS AND METHODS

CaP solids. Amorphous calcium phosphate (CaP_{am}) was synthesized according to the method of Termine and Eanes (*12*). Its amorphous character was verified by cylindrical x-ray diffraction techniques using a cylindrical camera.* The character of the crystalline CaP, commercially obtained was also verified by x-ray diffraction. Both the CaP_{cr} and CaP_{am} were dissolved in 6 *N* HCl and analyzed for calcium by atomic absorption, and for phosphorus by the method of Lucena-Conde and Pratt (*13*).

Preparation of C-14 diphosphonates. The 1-hydroxy[1-^{14}C]ethylidine diphosphonate (C-14 HEDP) was synthesized from the reaction of [^{14}C] acetic acid and phosphorous oxide (P_4O_6) to yield C-14 HEDP acid, which was converted to the sodium salt.

The [^{14}C]methylenediphosphonate (C-14 MDP) was

synthesized by reacting [^{14}C]methylenedibromide with triisopropylphosphite to yield the tetraisopropyl ester of C-14 MDP. The isopropyl groups were then hydrolyzed off with HCl to yield C-14 MDP acid, which was neutralized with NaOH to yield the sodium salt.

The [^{14}C]hydroxymethylene diphosphonate (C-14 HMDP) was synthesized from the tetraisopropylester of C-14 MDP by chlorination with hypochlorite to the tetraisopropyl ester of dichloro[^{14}C]methylene diphosphonic acid, which was then pyrolized to the dichloro[^{14}C]methylene diphosphonic acid. Hydrolysis of this compound by NaOH yielded [^{14}C]carbonyldiphosphonate which was then reduced to C-14 HMDP (sodium salt) with hydrogen and a nickel catalyst.

After recrystallization, all three tracers were checked by thin layer chromatography (TLC) and reverse isotope dilution (RID) and were found to be >98% pure.

In vitro adsorption of C-14 diphosphonates on calcium phosphates. A suspension of CaP_{am} or CaP_{cr} (12.0 mg) each in 14 ml of water was agitated for 3 min. Then 1.0 ml of C-14 HEDP, C-14 MDP, or C-14 HMDP was added to achieve a diphosphonate concentration of 1.6×10^{-4} *M* in the final mixture before the adsorption reaction. This was allowed to proceed for either 5 or 30 min. Following reaction, the mixtures were filtered through 0.45-μ filters to separate the suspended solid CaP plus adsorbed C-14 diphosphonate from the aqueous phase. The reaction vessel was rinsed twice with 2 ml of water, and the rinse used to wash the CaP residue. Following the washes, a clean collection flask was attached and the CaP residue washed with 2-ml aliquots of 3 *N* HCl. Each acid wash was separately layered over the residue on the filter to allow dissolution of the residue. After the dissolved residue was suctioned through the filter into the collection flask, the filter was washed twice with 2 ml distilled water. Both filtrate and dissolved residue solutions were radioassayed for C-14 by beta scintillation counting to determine the distribution of C-14 diphosphonate between filtrate and residue fractions. Recoveries and distribution of C-14 diphosphonate per mg CaP and per mole of calcium were then calculated.

In vivo osteogenesis and uptake of Tc-99m-labeled diphosphonates. Acid-insoluble bone matrix (AIBM) from the femurs and tibias of mature Sprague-Dawley rats (300–400 g) was prepared according to the method of Reddi (*11*).

Sixty Sprague-Dawley rats (190–210 g) were shaved from mid-torso down and anesthetized with methoxyflurane. Using sterile instruments a 1 cm incision was made in the thigh skin, then a subcutaneous pocket was created by blunt dissection with a mosquito hemostat. A No. 5 gelatin capsule containing 16.0 ± 0.5 mg of previously prepared AIBM powder was then inserted into the implant pocket. The incision was closed with wound clips. Each rat received two implants, in the right and left thighs. With the same procedure seven additional rats received sham implants, consisting of empty gelatin

TABLE 1. IN VITRO ADSORPTION OF C-14 DIPHOSPHONATES ON CALCIUM PHOSPHATES

System*	C-14-labeled diphosphonate	Reaction time (min)	Moles diphosphonate adsorbed/mole Ca ($\bar{x}$ ± s.d.)
Crystalline CaP	HEDP†	5	0.592 ± 0.012§
	MDP‡	5	0.583 ± 0.022
	HMDP‖	5	0.663 ± 0.008
	HEDP	30	0.717 ± 0.024
	MDP	30	0.730 ± 0.057
	HMDP	30	0.974 ± 0.033
Amorphous CaP	HEDP	5	0.910 ± 0.022
	MDP	5	0.868 ± 0.051
	HMDP	5	1.68 ± 0.021
	HEDP	30	1.37 ± 0.080
	MDP	30	1.24 ± 0.073
	HMDP	30	1.65 ± 0.022

* Crystalline CaP was calcium hydroxyapatite (molar Ca/P = 1.66); Amorphous CaP was amorphous by x-ray (molar Ca/P = 1.35); 12 mg of solid was used in each system; 2–6 systems each value. Recoveries were 98–101% on each system.

† 1-hydroxy[1-^{14}C]ethylidene diphosphonate (pH = 7.4).

‡ [^{14}C]methylenediphosphonate (pH = 7.4).

‖ [^{14}C]hydroxymethylenediphosphonate (pH = 7.4).

§ Groups within brackets are not significantly different. Groups in separate brackets are significantly different from each other ($p = 0.05$).

capsules, in the left and right thigh areas.

Fifteen days after implantation (at the earliest active osteogenic period), the rats were randomly assigned to treatment groups based upon body weights. In addition to the commercial HEDP‡ and MDP‖ skeletal imaging agents, the HMDP agent (*14*) was included in this study. Four vials of each bone agent were prepared according to the manufacturer's instructions using equal volumes of pertechnetate (Tc-99m) to achieve a final activity of ~75 mCi/vial. Greater than 98% tag efficiency was indicated by TLC. Five rats were injected from each vial, each rat receiving 50 μl intrajugularly of either Tc-99m HEDP, Tc-99m MDP, or Tc-99m HMDP. All sham-treated animals were injected from the vials of Tc-99m HEDP. Whole-body scintiphotos were obtained with an Anger camera at 3 hr after dose using the high-resolution collimator, followed by specific implant-area scintiphotos with a pinhole collimator.

After skeletal imaging, all animals from each group were killed at 3 hr after dose and implant "pellets" (isolated sites of osteogenesis), with an adjacent uninvolved area of undamaged skin and a blood sample were removed. Sham-operated sites were excised, along with an adjacent section of undamaged skin. All samples were radioassayed for Tc-99m with a gamma spectrometer. To determine uptake solely due to osteogenesis, comparisons were made between (a) implant and normal skin, (b) implant and sham, and (c) sham and normal skin. After the decay of the Tc-99m in each rat, the two pellets from the thigh were combined, the tissue from the two sham sites were combined, and the two normal skin samples were combined. After perchlorate wet oxidation of each combined sample (*15*), calcium content was measured by atomic absorption.

RESULTS

In vitro adsorption of C-14 diphosphonates on calcium phosphates. The adsorption of C-14-tagged HEDP, MDP, and HMDP on crystalline and amorphous CaP is shown in Table 1. The C-14 HMDP adsorbed at a significantly higher level than either the C-14 MDP or C-14 HEDP, whether with 5 or 30 min reaction or on crystalline or amorphous CaP. The highest uptake for C-14 HMDP was seen on the CaP_{am}; in this case, the reaction with CaP_{am} appeared to be complete at only 5 min whereas with C-14 MDP and C-14 HEDP a significant increase in adsorption between 5 and 30 min reaction occurred. No significant differences in adsorption of phosphonate were seen between C-14 MDP and C-14 HEDP on CaP_{cr} at either time or on CaP_{am} after 5 min of reaction. There was a small but significantly larger adsorption of C-14 HEDP on CaP_{am} than of C-14 MDP at 30 min. All three C-14 phosphonates had a significantly higher adsorption on CaP_{am} than on CaP_{cr}. Interestingly, the CaP_{am} had a lower surface area (41 m^2/g) than the CaP_{cr} (52 m^2/g) as measured by the nitrogen adsorption method (Strohlein Area Meter).

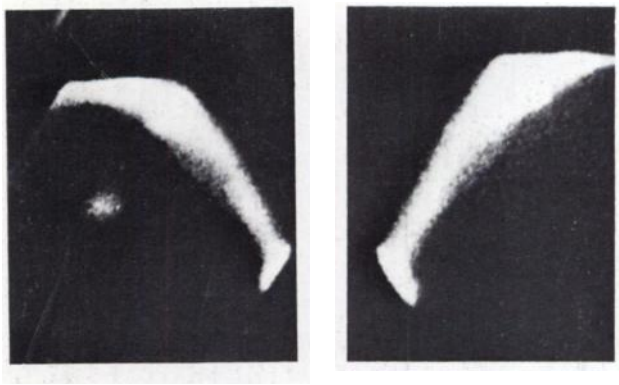

FIG. 1. Scintiphotos of sham implant site in thigh (left) and bone-matrix implant (right) at 3 hr after dose. Scintiphoto on right shows localized uptake of Tc-99m bone agent at site of induced osteogenesis just below high-uptake area in knee joint (also an active osteogenic site).

In vivo osteogenesis and uptake of Tc-99m-labeled diphosphonates. Representative scintiphotos of the implant sites in the animals, obtained at 3 hr after dose, are shown in Fig. 1. The osteogenic area was visualized using the pinhole collimator placed over the implant site, and correlates well with the findings of Garcia (*16*) using a similar system (*17*). Corresponding sham implant sites were also scanned, but no accumulation of Tc-99m was

TABLE 2. TISSUE DISTRIBUTION OF Tc-99m IN RATS AT 3 HR AFTER DOSE

Tc-99m* diphosphonate	Tc-99m (% dose/g tissue)			
	Pellet†	Normal skin†	Sham†	Blood‡
HEDP	0.072 ± 0.008	0.022 ± 0.009	0.024 ± 0.010	0.020 ± 0.010
MDP	0.098 ± 0.031	0.023 ± 0.006	—	0.029 ± 0.005
HMDP	0.168 ± 0.048	0.012 ± 0.003	—	0.011 ± 0.004

* Each rat received 0.05 ml of the appropriate Tc-99m diphosphonate solution, intrajugularly.

†,‡ Groups within brackets are not significantly different; groups in separate brackets are significantly different from each other ($p = 0.05$). To give the above average values, there are 32–35 samples per pellet value, 19 to 20 per normal skin, 14 for sham, and 20 for blood.

TABLE 3. Tc-99m RETENTION IN OSTEOGENIC PELLET NORMALIZED TO TISSUE CALCIUM

Tc-99m-diphosphonate*	Tc-99m % dose/mg Ca†	
	Uncorrected for normal skin	Corrected for normal skin‡
HEDP	0.371 ± 0.031 (38)‖	0.104 ± 0.086
MDP	0.410 ± 0.095 (38)	0.139 ± 0.102
HMDP	0.774 ± 0.270 (39)	0.639 ± 0.253

* 0.05 ml of the appropriate Tc-99m diphosphonate solution was given to each rat, intrajugularly.

† Groups within brackets are not significantly different; groups in separate brackets are significantly different at p = 0.05.

‡ For each rat the % Tc-99m/mg Ca in normal skin was subtracted from the % Tc-99m/mg Ca in osteogenic pellet, and data analyzed.

‖ Parentheses give number of separate samples.

seen at the sham site (Fig. 1, left). At the osteogenic site (implantation of AIBM), the "pellets" produced were physically uniform from animal to animal. Most were oval or round, were firm, and were easily recognized and freed from surrounding tissue.

The results of Tc-99m radioassay of pellets, normal skin, sham sites, and blood at 3 hr after injection of Tc-99m HEDP, Tc-99m MDP, or Tc-99m HMDP are shown in Table 2. Osteogenic site (pellet) uptake and retention of Tc-99m was significantly higher than in normal skin or sham sites for all groups. Rats administered Tc-99m HMDP had significantly higher pellet uptake of Tc-99m than corresponding animals given Tc-99m HEDP or Tc-99m MDP. There was no significant difference in uptake between the last two agents. Comparison of Tc-99m blood values at 3 hr after dose with the diphosphonate bone agents, showed that Tc-99m HMDP had the lowest blood retention followed by Tc-99m HEDP and Tc-99m MDP, which were not significantly different.

Calcium concentration in the osteogenic pellets was significantly higher (282 ± 62 μg Ca/g sample) than in either the sham (103 ± 82) or normal skin samples (90.1 ± 20). Calcium concentrations in pellets among treatment groups was not significantly different. Technetium-99m uptake in the osteogenic pellets was compared among the three bone agents after normalizing the Tc-99m uptake to calcium present in the tissue. The results of these comparisons are shown in Table 3. Pellet uptakes of Tc-99m HEDP and Tc-99m MDP normalized to calcium were not significantly different, but uptake into pellets from the Tc-99m HMDP group, normalized to calcium, was significantly higher than that for either of the other two agents whether or not corrected for normal skin Tc-99m uptake (Table 3).

DISCUSSION

Sites of new bone formation or hyperactive metabolic bone (such as encountered in Paget's disease and embryonic chick bone) are known to have high levels of CaP_{am} (*5,7*). The higher uptake of phosphonates on CaP_{am} than on CaP_{cr} (Table 1) in spite of the smaller surface area of the former was a surprise and suggests that it is the growing face (001) of the apatite that provides the selectivity of adsorption for the specific phosphonate. That is, apatite at first grows rapidly along the (c) axis to give long needle-like crystals, thus newly nucleated apatite will have a much higher ratio of rapidly growing face (001) to other crystal faces when it is first formed.

At 30 min, when the adsorption reaction is complete (*18*), one should compare *differences* in adsorption per mole of calcium (Table 1): between adsorption on CaP_{am} (as in a bone lesion, i.e., target) and adsorption on CaP_{cr} (as in mature bone, i.e., nontarget). It then becomes clear that both HEDP and HMDP have the greatest difference. This CaP_{am} to CaP_{cr} difference agrees with clinical observations for both HEDP (*19,20*) and HMDP (*21*) in terms of the tumor-to-bone ratio of Tc-99m. These two were equal in lesion detection (*22*), but HMDP was significantly superior to HEDP in overall scan quality (*22*) as might be expected from the present in vitro and in vivo blood clearance (*14*) data. The highest affinity for both crystalline and amorphous CaP combined with the structural specificity (OH-group) of tridentate adsorption at the 001 face of hydroxyapatite (*23,24*), low solubility of the calcium salt of HMDP (*18*), as well as the rapid clearance of blood and the low soft-tissue Tc-99m levels make this single agent (HMDP) a preferred diphosphonate for skeletal imaging with Tc-99m. In the clinic these properties of HMDP translate to either lower doses of Tc-99m to the patients or to shorter scan times for equal sensitivity to HEDP for focal skeletal abnormalities.

FOOTNOTES

* Rigaku Rotaflex

† Stauffer Chemical Co.

‡ Osteoscan, Procter & Gamble Co.

‖ Osteolite, New England Nuclear, North Billerica, MA.

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